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Interleukin-8 and polymorphoneutrophil leucocyte activation in hemolytic uremic syndrome of childhood

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Interleukin-8 and polymorphoneutrophil leucocyte activation in hemolytic uremic syndrome of childhood. Polymorphoneutrophil leucocytes (PMNLs) are implicated in the pathogenesis of diarrhea-associated hemolytic uremic syndrome (D+ HUS). We investigated mechanisms of PMNL involvement by measuring tumor necrosis factor α (TNFα) and the novel cytokine, interleukin-8 (IL-8), a potent activator of neutrophils, together with α1- antitrypsin-complexed elastase (α1-AT-E) as a marker of neutrophil degranulation, and anti-neutrophil cytoplasmic antibodies (ANCA). IL-8 was not detected in the 17 normal children, but was significantly elevated in 20 of 25 D+ HUS children ($P < 0.005$), and in three of nine children with non-diarrhea-associated (D−) HUS. Sequential data showed that IL-8 peaked transiently in the circulation, reaching a maximum just before a more protracted burst of α1-AT-E. The IL-8 levels correlated significantly with circulating α1-AT-E concentrations ($r = 0.50$, $P < 0.05$). In D+ HUS IL-8 levels also correlated with the PMNL count ($r = 0.63$, $P < 0.005$), and the highest values were seen in those children who died in the acute phase of the disease. TNFα was raised in only 1 of 16 D+ HUS children and in no patients were ANCA detected. The data suggest that PMNLs in HUS are recruited by IL-8, that this cytokine plays a key role in the PMNL activation which occurs, and that agents which suppress this recruitment and activation might play a therapeutic role in this disorder.

There is now substantial evidence pointing to PMNL involvement in the pathogenesis of D+ HUS. This disorder, which is the most common cause of acute renal failure in children in the United Kingdom, is characterized by hemolytic anemia, thrombocytopenia and renal failure [1, 2]. Two broad subtypes are recognized: the first is associated with diarrheal prodrome (D+) whereas the second, which is rare in childhood and has a worse prognosis, is not (D−) [2]. The peripheral blood PMNL count is raised at presentation in D+ HUS [3], and there is an association between the height of the PMNL count, acute mortality, and residual nephropathy [3–6]. There is evidence that activated PMNLs cause endothelial injury [7–9] and that their release products can damage the glomerular basement membrane [10]. In D+ HUS children, PMNLs have been found to adhere more avidly to endothelium and to damage the endothelial cell [11], and alpha 1-antitrypsin complexed elastase (α1-AT-E) levels are raised at presentation, reflecting PMNL activation and degranulation [12].

The aim of the present study was to investigate mechanisms of PMNL involvement by measuring mediators and markers of PMNL activation in the serum and plasma of children with D+ HUS. We measured two key participants in the cytokine cascade: tumor necrosis factor α (TNFα) and interleukin 8 (IL-8). TNFα is a proinflammatory cytokine and a proximal mediator in the cascade having the ability to induce gene expression for other cytokines and receptors such as IL-1, IL-6, IL-2 receptor and IL-8 [13, 14]. IL-8 is a cytokine which is produced by monocytes and is primarily defined as a selective activator and chemoattractant of PMNLs; it stimulates the release of lysosomal enzymes and superoxides from them [15]. In this study we investigated whether IL-8 was released into serum in the acute phase of D+ HUS and its relationship to peripheral blood PMNL count; we measured PMNL α1-AT-E which is a marker of PMNL degranulation [16–18] and anti-neutrophil cytoplasmic antibody (ANCA) which is present in other vasculopathies [19–22].

Methods

Patients

Thirty-four children with HUS treated at the Hospital for Sick Children, Great Ormond Street, London, were studied. The criteria for diagnosis were a microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. They were divided into two groups on the basis of the presence (D+ HUS; $N = 25$) or absence (D− HUS; $N = 9$) of a diarrheal prodrome. Three of the children with D+ HUS died within four days of admission to the Unit. Blood samples were taken within 24 hours of admission, and nine children also had serial samples taken throughout the acute phase of their illness.

Blood samples were also obtained from: (1.) seventeen healthy control children admitted for routine surgical procedures or investigation of short stature; and (2.) fifteen sick children identified by the hematology laboratory as having a PMNL count $>10 \times 10^9$/liter, designated high PMNL count controls, they had a septicemia or other infection, and all had a normal plasma creatinine concentration. Blood samples were obtained from them within 48 hours of admission. (3.) Ten children with chronic renal failure were included, all of whom

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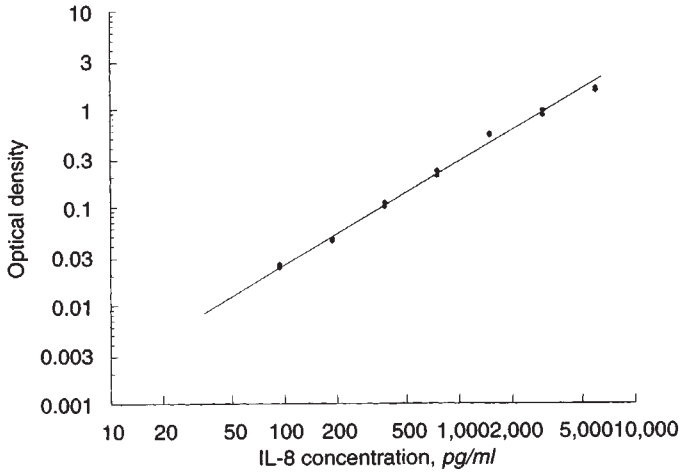

Fig. 1. *IL-8 standard curve - plotting IL-8 concentration (pg/ml) against optical density (OD).* The minimum detectable dose of IL-8 is 50 pg/ml.

who had a glomerular filtration rate (GFR) less than 15 ml/min/1.73 m^2 SA.

Samples

Blood samples were collected for measurement of IL-8, TNFα, and ANCA into plain tubes with no anticoagulant and for α1-AT-E into potassium EDTA (ethylenediaminetetraacetic acid) tubes. The samples were separated by centrifugation at 2000 g within 30 minutes, and serum and plasma were stored at −70°C until assayed.

Methods

Serum IL-8 immunoassay

IL-8 was measured by solid phase enzyme linked immunoassay (ELISA; Quantikine, Research and Diagnostic Systems, Minneapolis, Minnesota, USA). A monoclonal antibody specific for IL-8 was coated onto the microtiter plates provided. Standards with known amounts of IL-8 and samples were pipetted, in duplicate, into the wells, and any IL-8 present bound by the immobilized antibody. For detection, an enzyme-linked polyclonal antibody specific for IL-8 was applied. A substrate solution containing tetramethylbenzidine and H_2O_2 was added, and the color developed was proportional to the amount of IL-8 bound in the initial step. A standard curve was drawn of optical density (OD) against IL-8 concentration (Fig. 1). The lower limit of detection in this assay was 50 pg/ml. This was determined by adding 2 standard deviations to the mean optical density value of 5 zero standard replicates and calculating the corresponding concentration from the standard curve. The intra-assay coefficient of variation (CV) was 8% (N = 10) and the interassay CV was 9.3% (N = 5).

Serum TNFα immunoassay

TNFα was measured by solid phase ELISA (Quantikine). A monoclonal antibody specific for TNFα was coated onto the microtiter plates provided. Standards with known amounts of TNFα and samples were pipetted, in duplicate, into the wells. For detection, an enzyme-linked polyclonal antibody specific for TNFα was applied. A substrate solution was added, as above, and a standard curve drawn of OD against the concentration of TNFα in the standard wells. The lower limit of detection for this assay was 10 pg/ml. The intra-assay CV was 5.4% (N = 10) and inter-assay CV 4% (N = 5).

Human α1-AT complexed elastase immunoassay

Plasma α1-AT-E was measured by a modified ELISA as previously described [12]. A sheep anti-human elastase antibody (ICN Immunobiologicals, High Wycombe, UK) specific for leucocyte elastase was coated onto microtiter plates (Nunc, Roskilde, Denmark). For detection, horseradish-peroxidase-conjugated sheep anti-human α1-AT (The Binding Site Ltd., Birmingham, UK) was applied. A substrate was added and the color developed in proportion to the amount of α1-AT complexed elastase bound in the initial step. Standards were obtained by adding increasing amounts of human elastase (Sigma, Poole, Dorset, UK) to a constant volume of control plasma, giving final elastase concentrations of 50 to 16,000 ng/ml. A standard curve was prepared of OD versus α1-AT complexed elastase in the standard wells and by comparing the OD of the samples to the standard curve of the concentration of α1-AT complexed elastase in the unknown samples determined.

Immunoassay for ANCA against an acid extract of neutrophil cytoplasm

IgM and IgG ANCA were measured in serum by an ELISA technique using acid extracted neutrophil cytoplasm antigen prepared as described by Savage et al [23]. For each preparation, the optimal dilution for coating was determined using negative control sera and positive patient sera. The antigen was coated onto microtiter plates (Nunc). Test or control serum samples were added in duplicate to neutrophil antigen-coated plates. For detection, peroxidase conjugated anti-human IgG and IgM (Sigma) were applied, and after incubation and washing a substrate was added. After further incubation the reaction was stopped and ODs measured using an ELISA reader. The OD values of the standard sera at various dilutions were used to construct a standard curve of arbitrary units, from which the ANCA content of each test serum could be estimated. The arbitrary units were set as 64 and 32 for the IgG class ANCA-positive standard serum and IgM class ANCA-positive standard serum, respectively. The normal range for IgM for this assay is 0.5 to 9.1 units and for IgG 0.5 to 9.0 units.

Statistical analysis

Fisher's Exact test was used to determine the significance of the IL-8 results in the different groups compared to normals. The Mann-Whitney test was used as a test of significance of difference between the medians of the different groups. The correlations between IL-8 and α1-AT-E, and IL-8 and PMNL count were examined non-parametrically using Spearman's rank correlation coefficient. α1-AT-E and PMNL counts were log-normally distributed, and geometric means were compared using Student's t-test on log-transformed data.

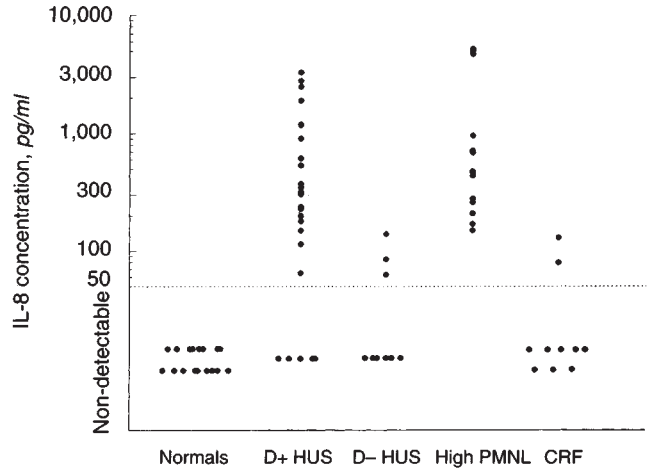

Fig. 2. *IL-8 concentrations in D+ HUS, D− HUS, high polymorphoneutrophil leucocyte (PMNL) count controls and chronic renal failure (CRF).* The dotted line corresponds to the minimum detectable dose and all points below this line represent undetectable levels.

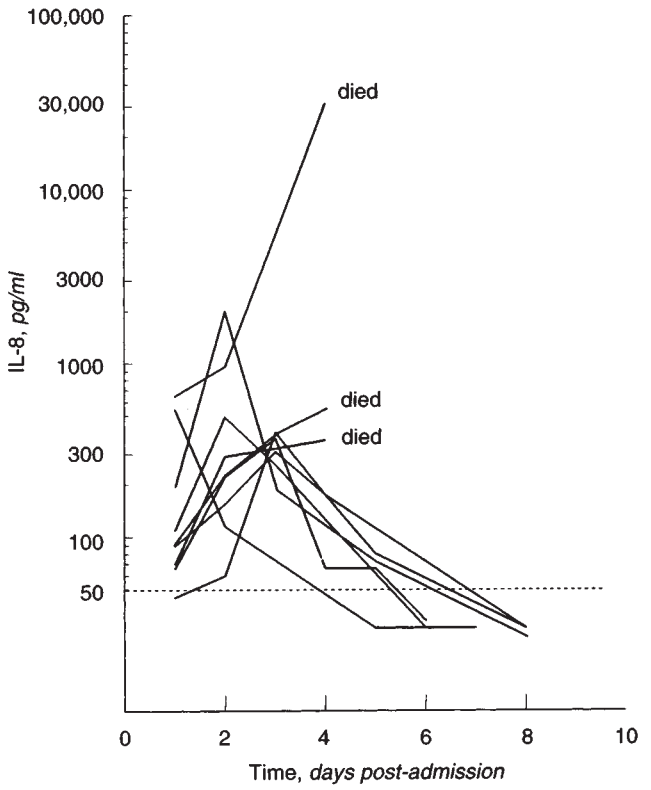

Fig. 3. *Serial IL-8 levels (pg/ml) in 9 children with D+ HUS following admission.*

Results

Serum IL-8

IL-8 was not detected in the serum of 17 normal healthy children. It was, however, detected and was significantly elevated in 20 of 25 children with D+ HUS [median value: 305 pg/ml, range: non-detectable (ND) - 3300, $P < 0.005$], and in three of nine D− HUS children ($P < 0.05$; Fig. 2). The median IL-8 value in D+ HUS was significantly higher than in D− HUS ($P < 0.005$). Only two of ten children in chronic renal failure (CRF) had detectable IL-8 levels, and this did not reach significance ($P > 0.05$). Children in the high PMNL control group had significantly elevated IL-8 with a median value of 460 pg/ml (range 150 to 5200, $P < 0.005$), and these results were not significantly different from those obtained in the D+ HUS group ($P > 0.05$).

Serum TNFα

When 40 normal donor sera were evaluated using this assay (Quantikine) the maximum observed value was 25.8 pg/ml. TNFα was detected in the serum of only 1 of 16 of the children with D+ HUS, in whom the level was 1800 pg/ml. Moderately elevated levels were detected in three of 15 high PMNL controls with values of 27, 28, and 30 pg/ml recorded, and no significant activity was detected in any of the normal children or those with D− HUS.

Serum ANCA

No IgM ANCA was detected in any of the 10 D+ HUS or 9 D− HUS sera tested. One of the D− HUS group had a weakly positive IgG ANCA of 13.1 units; the remaining 8 D− and all the D+ HUS children had negative IgG ANCA results.

Serial IL-8 and α1-AT-E

Serial measurements of IL-8 were undertaken in nine children with D+ HUS following their admission in acute renal failure, and in seven of them α1-AT-E was also measured. This data is graphically illustrated in Figures 3 and 4. In this group of patients the serum IL-8 reached a maximum on day 3 with a mean of 8,193 pg/ml, and then fell sharply to mean values of 272 and 312 pg/ml on days 4 and 5, respectively. α1-AT-E peaked on days 3 to 4 with mean values of 5,100 and 4,200 ng/ml, respectively, but then declined more gradually. The highest values for both IL-8 and α1-AT-E were seen in the three children who died in the acute phase of the disease (Figs. 2 and 3). We found a significant positive correlation between the IL-8 and the circulating α1-AT-E levels ($r = 0.50$, $P < 0.05$) in these children.

Polymorphoneutrophil leucocyte counts (PMNLs)

The children with D+ HUS had a raised PMNL count at presentation with a geometric mean of 14.0×10^9/liter (range 7.6 to 24.3). Their mean PMNL count was significantly higher than the PMNL count of 4.9×10^9/liter (2.7 to 6.8) in normal controls ($P < 0.001$) and 5.7×10^9/liter (4.6 to 7.2) in D− HUS ($P < 0.001$). Serial PMNLs on eight of the nine patients are illustrated in Figure 5; they remain elevated over a longer period than both the IL-8 and the α1-AT-E, returning gradually into the normal range by days 11 to 12.

There was a significant positive correlation between the PMNL count and the IL-8 level at presentation in the D+ HUS group ($r = 0.63$, $P < 0.005$), but not in the high PMNL group ($r = 0.34$, $P > 0.05$).

Discussion

The etiology of D+ HUS is more clearly understood now that an association has been found with toxin-producing enteric

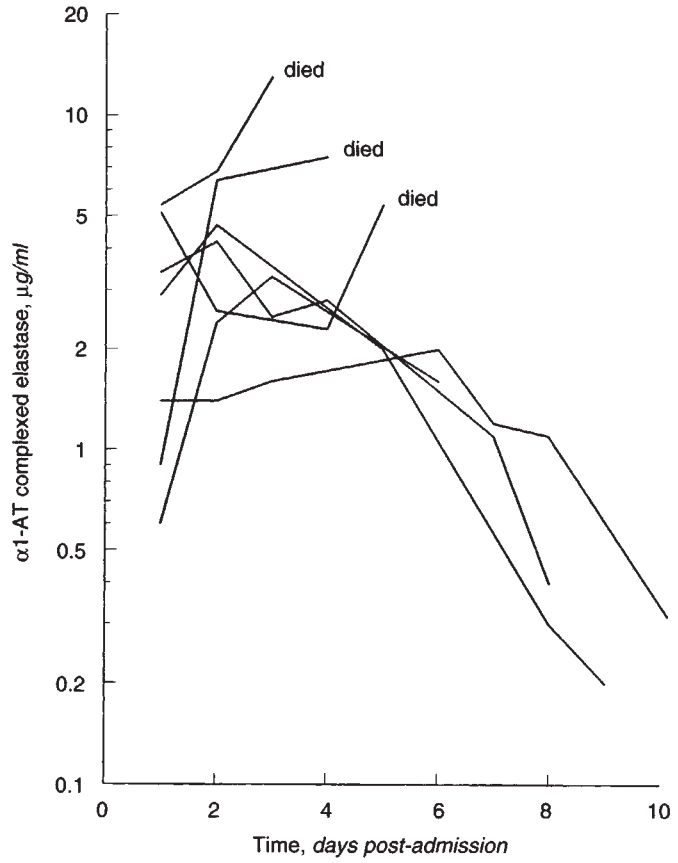

Fig. 4. *Serial plasma α1-antitrypsin complexed elastase levels (μg/ml) in 7 children with D+ HUS following admission.*

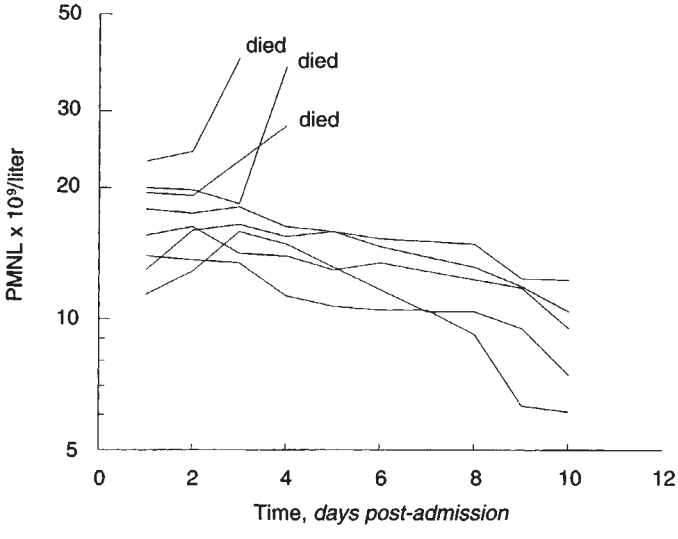

Fig. 5. *Serial peripheral blood polymorphoneutrophil leucocyte (PMNL) counts in 8 children with D+ HUS following admission.*

pathogens, in particular Verocytotoxin-producing *Escherichia coli* (VTEC) [24, 25]. It is also well recognized that damage to the glomerular endothelium is the main site of injury in HUS, and there is now increasing evidence suggesting that the PMNL

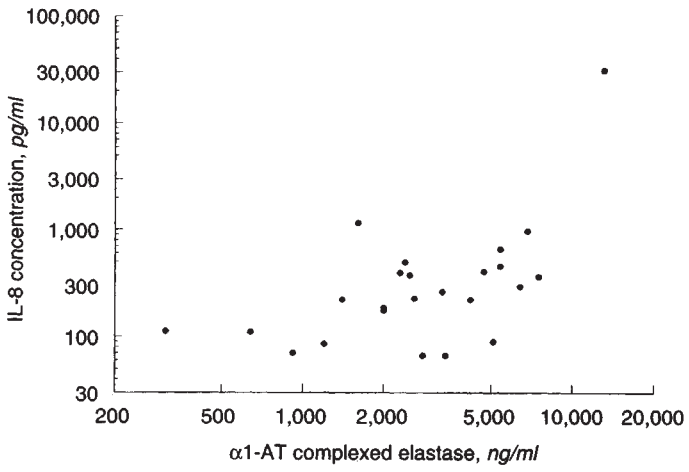

Fig. 6. *Graph illustrating the correlation between IL-8 (pg/ml) and α1-AT-E (ng/ml).* r = 0.50; *P* = 0.02.

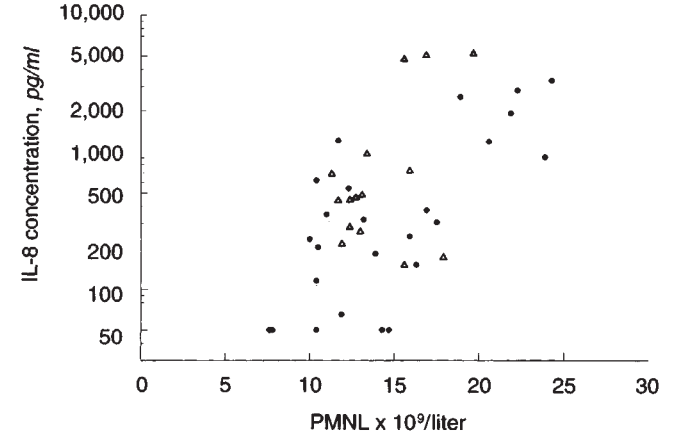

Fig. 7. *IL-8 levels and peripheral blood polymorphoneutrophil leucocyte (PMNL) counts in D+ HUS (●; r = 0.63;* P = *0.002) and high PMNL count controls (△; r = 0.34;* P = *0.21).*

has an important role in the pathogenesis. There is a significant relationship between the PMNL count at onset and adverse outcome [3–6]. HUS PMNLs show increased adhesion to endothelium in vitro [11], α1-AT complexed elastase levels are increased in the acute phase of the syndrome [12], and by electron microscopy peripheral blood neutrophils appear degranulated [26].

It is not known whether the neutrophil response which occurs is attributable to the action of verocytotoxin or to endotoxin, the lipopolysaccharide (LPS) derived from the bacterial cell wall. Endotoxin has been suggested as having a pathogenic role in D+ HUS but has not yet been studied in VTEC disease. It alters the surface characteristics of endothelial cells via a cascade of cytokines which renders them adhesive for neutrophils [27, 28].

Significant TNFα was detected in only one of the 16 D+ HUS children tested; this child was admitted in the early phase of her disease before the onset of acute renal failure. She subsequently went into renal failure and developed neurological complications. She survived her acute illness but has residual nephropathy with proteinuria and a reduced GFR. TNFα is a proximal

mediator in the cytokine cascade, appearing in the circulation of several species as a brief, early peak after infusion of bacteria or bacterial LPS [13]. The majority of our patients were referrals from other hospitals admitted to the unit several days after the onset of their illness, which may account for our inability to detect TNFα in the others at the time of their admission.

IL-8 is a recently described 6-10 kDa protein known for its in vitro leucocyte chemoattractant and activation properties [29–36], and is a potential mediator of host response to injury and infection. It has several properties that suggest that it plays a role in mediating some of the inflammatory responses of neutrophils induced by endotoxin, IL-1 and TNFα [15]. It is known to cause shedding and up-regulation, respectively, of the two important neutrophil adhesion molecules: leucocyte adhesion molecule 1 (also known as LAM-1, LECAM-1 and L-selectin) and CD11b/CD18 [37–39]. Adherence via the latter has been shown to mediate neutrophil priming for the respiratory burst [40] and degranulation [41]. It has been shown that IL-8 appears in the circulation of primates in vivo during septic shock, sublethal endotoxemia, and after the administration of IL-1α [42]; more recently increased levels have been detected in normal humans as part of the acute inflammatory response to intravenously administered endotoxin [43].

We detected significantly elevated IL-8 in both D+ and D− HUS and in high PMNL controls. IL-8 levels were significantly higher in D+ than in D− HUS. Our study therefore demonstrates that IL-8 is produced in vivo as part of the inflammatory response occurring in humans during the acute phase of D+ HUS. The sequential data on nine children with D+ HUS shows that IL-8 circulates in blood with peak levels occurring at 72 hours.

Serial measurements of α1-AT-E, a known marker of PMNL activation and degranulation [16–18], were undertaken on seven of the nine patients. Plasma α1-AT-E levels peaked at 72 to 96 hours and declined more slowly than the corresponding IL-8 levels. From this data IL-8 appears to peak more transiently in the circulation, achieving a maximum level just before the more protracted burst of complexed elastase. The correlation of IL-8 with α1-AT-E suggests that IL-8, which is known to stimulate degranulation of adherent neutrophils, may be promoting the release of this proteolytic enzyme in these patients. A positive correlation was also found between IL-8 and the PMNL count at presentation in the D+ HUS group. This correlation is of particular importance when considering that the outcome is associated with the PMNL count at presentation [3–6], and indeed the higher levels of IL-8 were recorded in three patients who died during the acute phase of the disease.

From our data we cannot conclude whether the observed release of IL-8 into serum in D+ HUS is induced by Verocytotoxin or high LPS levels. It is also unknown whether serum IL-8 represents a pathogenetically important mechanism or is a concommitant marker of disease activity. There is, however, increasing evidence suggesting that IL-8 promotes leucocyte adhesion in vivo and leads to recruitment of PMNLs to sites of tissue inflammation [37, 38]. IL-8 has previously been shown to induce granulocytosis upon systemic injection and skin reacton upon local injection in experimental animals [44]. The recruitment of PMNLs to inflammatory sites is also dependent on other chemotactic factors generated in tissue, such as the cleavage products of complement activation C5a and C3b, which we are currently measuring prospectively in children with HUS. Elevated elastase levels do indicate that granulocytes become highly activated in this disease and may represent a source of endothelial cell damage. Our findings in this study are consistent with the hypothesis that IL-8 contributes to the dynamics of circulating PMNLs and acts as a mediator of the PMNL activation which occurs. We conclude that IL-8 participates in the complex cascade of cytokine responses to infectious and inflammatory stimuli, and plays a significant role in the pathophysiology of D+ HUS. These findings raise the possibility that agents which suppress PMNL recruitment and activation may have a therapeutic role in this disorder.

We were unable to detect significant ANCA activity in HUS serum and conclude that this antibody is not implicated in this disease.

Acknowledgments

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